Supplementary File 1

Patient recruitment

Forty-two patients with active inflammatory arthritis naive to biologic treatment were recruited from outpatient clinics at the Department of Rheumatology, St. Vincent's University Hospital. At baseline, 50% of the patients had not taken disease-modifying anti-rheumatic drugs (DMARDs) or steroids. Those who were receiving DMARDs were taking methotrexate (MTX) alone (35%), MTX plus sulfasalazine (10%), or hydroxychloroquine (5%). All patients, including those taking DMARDs, had active inflammation of one or both knees at the time of assessment. Clinical disease activity was assessed with the 28-joint Disease Activity Score using the C-reactive protein level (DAS28-CRP). Ethical approval to conduct this study was granted by St. Vincent's Healthcare Group Medical Research and Ethics Committee.

Arthroscopy, oxygen partial pressure measurements, and sample collection

Under local anesthesia, each patient underwent arthroscopy of the inflamed knee, performed using a Wolf 2.7-mm needle arthroscope. Macroscopic vascularity was quantified under direct visualisation at arthroscopy using a well-established visual analogue scale (VAS) 1-100mm.[5] A Licox combined pO_2 and temperature probe (Integra Life Sciences, UK) was used to determine oxygen partial pressure as previously described.[11] A subgroup of patients (n=19) underwent a second arthroscopy three months after commencement of TNFi. Patients were categorised according to remission criteria using the DAS28 cut-off $<$ or $>$ 2.6, where patients with DAS28-CRP<2.6 were defined as responders (n=6) and patients with DAS28-CRP>2.6 were defined as non-responders (n=13). Synovial membrane biopsy samples were obtained from the site of the oxygen tension measurement and immediately fixed in 10% formalin and paraffin embedded or embedded in OCT mounting media for

immunohistochemical analysis or snap frozen in liquid nitrogen for mitochondrial mutagenesis analysis. Matching serum and synovial fluid samples were collected immediately before arthroscopy and stored at −80°C.

Cell Culture

Primary RASF were isolated from RA biopsies by digestion with 1 mg/mL collagenase type 1 (Worthington biochemical, Freehold, New Jersey, USA) in RPMI 1640 containing 10% fetal bovine serum (FCS) (Life Technologies-BRL, Paisley, UK) for 4 h at 37°C in humidified air with 5% CO_2. Dissociated cells were grown to confluence and used between passages 4 and 8. An immortalised normal human synoviocyte cell line (K4IM) were cultured in RPMI 1640 containing 10% fetal bovine serum (FCS). HMVEC (Lonza Wokingham, Berkshire, UK) were incubated in cell basal medium (EBM) supplemented with endothelial growth medium (EGM)-microvascular bullet kit containing 25ml FCS, 0.5ml hEGF, 0.5ml hydrocortisone, 0.5ml gentamicin and 0.5ml bovine brain extract. To examine the effect of hypoxia on mitochondrial function and cellular metabolism, RASF were seeded onto 96 well plates and into T25 flasks, cultured for 6 hrs with 1mM dimethyloxalylglycine (DMOG), an inhibitor of prolyl hydroxylases enzymes (Sigma-Aldrich, UK) or DMSO (1mM) vehicle control. MtDNA mutations, total levels of cellular ATP, ROS production, mitochondrial membrane potential (MMP) and mitochondrial mass (MM) were assessed. To examine the effect of glycolytic inhibition further experiments were performed with glycolytic inhibitor 3PO (20µM) or DMSO vehicle control (20µM).

***In vitro* mitochondrial dysfunction**

After 6-hour incubation with DMOG (1mM), total levels of cellular ATP, ROS production, mitochondrial membrane potential and mitochondrial mass were assessed. Luminescent ATP Detection Assay Kit (Abcam, UK) was used for quantitative detection of total levels of

cellular ATP. RASF were seeded into white 96-wells plate at the density of $1.5x10^4$ cells/well and allowed to attaching overnight. RASF were subsequently stimulated with DMOG, lysis solution was added into the cell suspension to lyse the cells and stabilize the ATP. After 5 mins substrate solution was added into each well, shaken for 5 mins, left in the dark to adapt for 10 mins before the luminescence was measured. To determine cellular ROS release from RASF in the presence of DMOG, DCFDA Cellular ROS Detection Assay Kit (Abcam, UK) was used. RASF were seeded into clear bottom, dark sided 96-well plates at a density of $2.5x10^4$ cells/well and allowed to attach overnight. Cells were washed in 1X buffer and stained with 25µM DCFDA in 1X buffer for 45 min at 37°C and 5% CO_2. After staining, cells were washed, treated with DMOG and incubated at 37°C in 5% CO_2. JC-1 Assay Kit (Abcam, UK) and Green-Fluorescent MitoTracker dye (Invitrogen, Ireland) were used to determine MMP and MM of RASF in the presence of DMOG, respectively. Cells were seeded into clear bottom, dark sided 96-well plates at the density of $1.5x10^4$ cells/well and allowed to attach overnight. For MMP, cells were washed in 1X dilution buffer and stained with 20µM JC-1 in 1X Buffer for 10 min at 37°C in 5% CO_2. After staining, cells were washed, treated with DMOG and incubated at 37°C in 5% CO_2. For MM, cells were stimulated with DMOG, then washed with PBS and stained with Green-Fluorescent MitoTracker dye for 45 min at 37°C in 5% CO_2. ROS, MMP and MM fluorescence signal was measured using the Spectra Max Gemini System with excitation and emission wavelengths of 485nm and 538nm, respectively. Mean fluorescence values from 4 wells for each condition were obtained.

Quantification of lactate levels in serum and synovial fluid from IA patients

Lactic acid levels were quantified in paired serum and synovial fluid from IA patients using a colorimetric assay (Biovision), and performed as per manufacturers instructions.

Transwell Invasion Chambers

Biocoat Matrigel Invasion Chambers (Becton Dickinson, UK) were used to assess RASF invasion in response to lactic acid (1mM), succinate (5mM) and 3PO (20µM) compared to vehicle control (20µM). Cells were seeded at a density of 2.5 x 10^4 per well in the migration chamber on 8µm membranes pre-coated with matrigel. RPMI containing lactic acid, succinate, 3PO or vehicle control were placed in the lower well of the chamber. Cells were allowed to migrate for 48 hrs in RPMI medium containing 1% FCS. Non-migrating RASF were removed from the upper surface by gentle scrubbing. RASF that had invaded were attached to the lower membrane were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. To assess the average number of invading cells, cells were counted in five random high power fields (hpf).

Quantification of pro-inflammatory mediators

To assess the effects of lactic acid, pyruvic acid and succinate on secretion of bFGF, VEGF, PIGF, IL-6, IL-8, RANTES, RASF were seeded in 96 well plates. Confluent RASF were serum starved for 24 hours and then cultured with lactic acid (1mM), pyruvic acid (1mM) or succinate (5mM) for 24 hrs. Supernatants were harvested and protein secretion levels quantified by MSD assays (Mesoscale Discovery, Rockville MD, USA) or specific ELISA (R&D). To assess the effect of 3PO on IL-6, IL-8, MCP-1, RANTES and Gro-alpha secretion, RASF and HMVEC were seeded in 96 well plates, grown to confluence and cultured under normoxic and hypoxic conditions in the presence of 3PO (20µM) or DMSO vehicle control (20µM). Supernatants were harvested and protein secretion levels were

measured by specific ELISA according to manufacturer's instructions (R&D systems, Minneapolis MN, USA).

Immunohistochemistry analysis and scoring

Immunohistochemistry was performed using synovial tissue and the DAKO ChemMate Envision Kit (Dako, Glostrup, Denmark). GAPDH, PKM2, GLUT 1, and ATP5B immunohistochemistry staining was performed using 3µm paraffin sections. Factor-VIII (blood vessels) immunohistochemistry staining was performed using 7µm cryostat sections. Paraffin embedded sections were baked for 30 minutes at 90°C, deparaffinised in xylene and rehydrated in alcohol and deionised water. Antigen retrieval was performed by heating sections in antigen retrieval solution (15ml of 1M sodium citrate and 15ml of 1M citric acid in deionised water, pH 6.0) in a pressure cooker. Slides were washed in PBS for 5 minutes. Cryostat sections were defrosted at room temperature for 20 minutes, fixed in acetone for 10 minutes and washed in PBS for 5 minutes. The following protocol was identical for paraffin and cryostat sections. Non-specific binding was blocked using 10% casein in PBS for 20 minutes. GAPDH (Trevigen, Gaithersburg, MD), PKM2 (Abgent, CA), GLUT 1 (Abcam, UK), ATP5B (Santa Cruz Biotechnology, CA) and Factor-VIII (DAKO) primary antibodies were incubated on sections for 2 hours at room temperature. An IgG1 control antibody was used as a negative control. Following primary antibody incubation, endogenous peroxidase activity was blocked using 0.3% H_2O_2. Slides were incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody (Dako). Color was developed in diaminobenzidine solution (1:50; Dako) and counterstained with hematoxylin. Slides were mounted in Pertex media and analyzed using an established and validated semiquantitative scoring method.[11, Supplementary Reference 1] Blood vessels were quantified by counting

the number of blood vessels per high powered field expressing Factor VIII, five random fields were analysed, and mean number calculated.[7] For glycolytic markers percentage positivity was graded on a well-established semi-quantification scoring method. 0–4 scale, where 0 = no stained cells, 1 = 1–25% stained cells, 2 = 25–50% stained cells, 3 = 50–75% stained cells, and 4 = 75–100% stained cells.[Supplementary Reference 1]

MRI Imaging Acquisition

A subgroup of the RA cohort underwent knee MRI prior to arthroscopy. All MR imaging was performed using a 1.5T MR imager (Sigma HDX; GE Medical Systems) between 24 and 72 hours prior to needle arthroscopy, to maximize the comparison between these investigations. A standardized acquisition protocol was previously developed to standardize the MRI image sequences and orientation. Briefly, patients were placed in the supine position with the target inflamed knee joint centralized in the magnetic field. A 20-gauge intravenous access line was inserted into an antecubital vein. Sixty seconds after the initiation of the dynamic MRI protocol, a bolus of a contrast agent (gadoteric acid; 0.1 mg per kg body weight) (Dotarem) followed by a 15-ml saline chase was delivered by hand injection. Images were acquired using a 3-dimensional T1-weighted spoiled gradient-echo dynamic sequence that resulted in 20 consecutive images of 20 sections with a temporal resolution of 22 seconds (repetition time 33 msec, time to echo for coronal MR sequences 15.7 msec, flip angle 45°, section thickness 2 mm, field of view 19 cm, matrix 256 × 192 pixels; axial orientation). Sagittal, coronal, and axial 2-dimensional sequences, including coronal proton density, coronal T2 with fat saturation, sagittal T2 with fat saturation, and sagittal T1 with fat saturation, were obtained after intravenous contrast administration. The total imaging time, including all of the procedures described above, was 45 minutes. MRI analysis was guided by preliminary studies that identified the most reliable sites for assessment, which included the medial and lateral parapatellar recesses, the intercondylar notch, and the suprapatellar pouch. Synovitis

was graded using a previously described validated semiquantitative scoring system (range 0–3, where 0 = normal synovium, 1 = diffuse, even thickening, 2 = nodular thickening, and 3 = gross nodular thickening of the synovium).[Supplementary Reference 2] The images were scored by a consultant musculoskeletal radiologist. A 4 regions (maximum possible total score 12) was calculated and used for analysis.

3PET/CT acquisition

Whole-body PET was performed following intravenous injection of 18F-FDG (5 MBq/kg) after patients had fasted for >6 h. Data acquisition was done in 3D mode 60 min after the injection using a PET-CT scanner (Biograph 16; Siemens Medical Solutions Inc., Munich, Germany). Patients were scanned from head to the toe in the arms-down position. Attenuation correction of the PET images was performed using CT, followed by reconstruction using an ordered subsets expectation-maximization algorithm into 128 × 128 matrices. PET images were interpreted by experienced nuclear physicians, and increased FDG uptake in both knees was compared. PET/CT and MRI knee images were fused by 3D volumetric analysis and anatomical collocation (JR).

Wound Repair Assay

RASF were seeded onto 48-well plates and grown to confluence. A single scratch wound was induced through the middle of each well with a sterile pipette tip. Cells were subsequently stimulated for 24 hrs with DMSO (20µM) and 3PO (20µM) under normoxic or 3% hypoxic conditions. RASF migration across the wound margins from 12 to 24 hrs was assessed and photographed using a phase-contrast microscope. Semi-quantitative analysis of cell repopulation of the wound was assessed. Briefly, images of the scratch wound assays were taken at X10 magnification. The mean closure of the wound was manually calculated from

the average of three individual measurements from each wound. This process was repeated for all technical replicates. Measurement of scratches at time 0 were designated as 100% open. From this, the percentage of closure for all scratches was calculated.

HMVEC tube formation

Matrigel (50µl) (Becton Dickinson, Mountain View, CA) was plated in 96-well culture plates after thawing on ice and allowed to polymerize for 1 hr at 37°C in humidified air with 5% CO_2. HMVEC were removed from culture, trypsinized and resuspended at a concentration of 4×10^4 cells/ml in EGM. Five hundred microliters of cell suspension was added to each chamber in the presence of DMSO (20µm) and 3PO (20µM) and cultured under normoxic or 3% hypoxic conditions. Endothelial cell tubule formation was assessed using phase-contrast microscopy and quantified as previously described.[15]

Quantification of lactate and glucose levels in RASF cultured under 3% hypoxia

RASF were cultured in 24 well plates and grown to confluence. RASF were exposed to 3% hypoxia or normoxia for 24hrs and supernatants harvested. Glucose and lactate levels were quantified using a Cobas 8000 analyzer using a standard Roche hexokinase method and a Radiometer ABL 800 series blood gas analyzer with a lactate specific electrode.

Transmission electron microscopy

RASF were grown to confluence in T75 flasks and were exposed to 3% hypoxia compared to normoxia for 24hrs. Following culture RASF were fixed with gluteraldehyde (3% in 0.05M Potassium Phosphate buffer, pH 6.8) for 1hr at room temperature. Samples were processed

and analyzed using a Jeol JEM2100 LaB6 (operated at 100 Kv). Digital images were obtained using an AMT XR80 capture system and ImageJ software.

Western Blot

K4IM and HMVEC were cultured under 3% hypoxia in the presence of DMSO (20µM) or 3PO (20µM) for 24 hrs. Following treatment protein lysates were prepared as previously described.[14,15] Protein (20µg-50µg) was resolved on SDS-PAGE (5% stacking, 10% resolving), gels were then transferred onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) prior to 1 hr blocking in wash buffer containing 5% non-fat milk with gentle agitation at RT. Membranes were incubated with rabbit polyclonal anti p-STAT3 (1:500; Cell Signalling Technology), total-STAT3 (1:500; Cell Signalling Technology), rabbit polyclonal anti Notch-1 (1:500; Millipore, Temecula, CA, USA) or mouse monoclonal anti-HIF1α (1:500, BD Biosciences, Oxford, UK) diluted in 5% non-fat milk containing 0.1% Tween 20 at 4°C overnight with gentle agitation. β-actin (1:5000, Sigma) was used as a loading control. Following three 15 min washes, membranes were incubated with appropriate horseradish peroxidase conjugated secondary antibodies (1:1000) for 3 hrs at RT. The signal was detected using SuperSignal® West Pico Chemiluminescent Substrate (Amersham Biosciences) and density of the bands was analyzed using EDAS 120 system from Kodak (Kodak, Rochester, NY, USA).

Statistical Analysis

SPSS15 system (SPSS Inc, Chicago, Illinois, USA) for windows was used for statistical analysis. Wilcoxon signed rank, Spearman's rank correlation coefficient and Mann-Whitney

U was used for analysis of non-parametric data. Student t-test was used for parametric data. p-values of less than 0.05 (*p<0.05) were determined as statistically significant.

Supplementary References

1. Youssef PP, Kraan M, Breedveld F, et al. Quantitative microscopic analysis of inflammation in rheumatoid arthritis synovial membrane samples selected at arthroscopy compared with samples obtained blindly by needle biopsy. Arthritis Rheum 1998;41(4):663-9.

2. Rhodes LA, Grainger AJ, Keenan AM, et al. The validation of simple scoring methods for evaluating compartment-specific synovitis detected by MRI in knee osteoarthritis. Rheumatology (Oxford) 2005;44(12):1569-73.